The Anti-Tumor Effect of Bee Honey in Ehrlich Ascite Tumor Model of Mice is Coincided with Stimulation of the Immune Cells

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Honey is thought to exhibit a broad spectrum of therapeutic properties including antibacterial, antifungal, cytostatic and anti-inflammatory activity and has been used for the treatment of gastric ulcers, burns, and for storage of skin grafts. The present study investigated the antitumor effect of bee honey against Ehrlich ascites tumor in mice and the possible mode of antitumor action. Peroral administration of mice with honey (10, 100 or 1000 mg/100 g BW) every other day for 4 weeks before intraperitoneal inoculation with Ehrlich ascites tumor (EAT, 1x10^6 cells) increased the number bone marrow cells as well as peritoneal macrophages, but not peripheral blood leukocytes nor splenocytes. The phagocytic function of macrophages as well as the T- and B-cell functions were also increased. Honey pre-treatment also recovered the total lipids, total proteins, as well as liver and kidney enzyme activities in EAT-bearing mice. In vitro studies on EAT cells demonstrated inhibitory effect of honey on tumor cell proliferation, viability % of tumor cells as well as the size of solid tumor. The present results indicate that the preventive treatment with honey is considerably effective against EAT in mice both in vivo and in vitro. The antitumor activity of honey may occur through the activation of macrophages, T-cells and B-cells.

Several types of immunopotentiators have been developed recently and are being studied for possible use in the treatment of patients suffering from malignant diseases (Block & Mead, 2003; Sunila & Kuttan, 2004). The increased interest in new approaches to the immunotherapy of cancer, and a considerable demand for therapeutic agents which can modulate the several forms of immunodeficiency have encouraged studies on the immunomodulatory mechanism of natural and synthetic substances (Mirandola et al., 2002; Valadares et al., 2003).

Honey is one of the most complex foodstuffs produced naturally, and certainly the only sweetening agent that can be used by humans without processing. Honey is produced by honeybees from carbohydrate-containing exudates produced by plants and contains all of the trace minerals that are essential to health (Terrab et al., 2004). A large number of organic compounds have been described as components of different types of honeys (Perez et al., 2002). The main components of honey volatiles belong, in general, to three principal categories such as terpenes, norisoprenoids, and benzene derivatives (D’Arcy et al., 1997). Honey is also characterized by containing different flavonoids (Siess et al., 1996). Experimental studies showed that flavonoids could inhibit carcinogenesis in rodents (Suschetet et al., 1990; Deschner et al., 1991). In addition, many flavonoids are known to modify the activities of drug metabolizing enzymes (Smith & Yang, 1994).

To explore its immunomodulatory properties, Duddukuri et al. (1997) reported that intraperitoneal administration of honey in low doses (100 µl) suppressed the induction of ovalbumin-specific murine humoral antibody responses. Honey also suppressed ovalbumin-specific immunoglobulin G (IgG) subclasses and other classes of antibody response (Duddukuri et al., 2001). T cell proliferation induced by antigens was significantly suppressed by various doses of natural and commercial honeys (Duddukuri et
The antitumor effect of bee honey in ehrlich ascite tumor is coincided with stimulation of the immune cells. On the other hand, Al-Waili & Haq (2004) reported that oral honey (0.8 g/kg of body weight/day) stimulated antibody production during primary and secondary immune responses against thymus-dependent and thymus-independent antigens. Karmakar et al. (2004) also found that honey significantly increased the humoral immunity in immunocompetent and immunodeficient mice. They confirmed the use of honey as an immunomodulator in experimental rodents. A recent study by Tonks et al. (2007) showed that a 5.8-kDa component, isolated from manuka honey could stimulate cytokine production from human monocytes. Honey potentiated the antitumor activity of chemotherapeutic drugs such as 5-fluorouracil and cyclophosphamide (Wattenberg, 1986). Studies by Gribel & Pashiniski (1990) indicated that honey possesses moderate antitumor and pronounced antimetastatic effects in five different strains of rat and mouse tumors. Rao et al. (1993) reported that caffeic acid derivatives, present in honey, inhibited azoxymethane-induced colonic preneoplastic lesions, which are relevant to colon carcinogenesis in male F344 rats. Honey might also prevent tumor implantation when applied locally (Hamzaoglu et al., 2000). Honey and Nigella grains together protected 100% against methylnitrosourea-induced oxidative stress and carcinogenesis (Mabrouk et al., 2002). Swellam et al. (2003) reported that bee honey is an effective agent for inhibiting the growth of both human and murine bladder cancer cell lines in vitro. Honey is also effective when administered intralesionally or orally in the murine bladder cancer cell line. Moreover, Orsolic & Basic (2004) found a pronounced antimetastatic effect by oral application of honey given before tumor cell inoculation. However, given after tumor cell inoculation, honey enhanced lung metastases. These findings indicate that honey activated immune system and may be advantageous with respect to cancer and metastasis prevention.

The main objective of the present study was to investigate the prophylactic effect of bee honey against Ehrlich ascites tumor in mice and the possible mode of antitumor action.

Materials and Methods

Animals

The experimental animals used in this study were female Swiss albino mice (8-10 weeks old, weighing about 20 g each). Mice were obtained from Helwan Research Animal Center, Cairo, Egypt, and were maintained in a quite room at 28°C. Mice received laboratory chow and water ad libitum and were allowed a period of 10 days, prior to the initiation of experiments, to acclimatize to the laboratory conditions.

Bee Honey

Bee honey (Research Institute of Plant Protection, Cairo, Egypt) was dissolved in distilled water, and doses of 10, 100 or 1000 mg/100 g body weight were orally administered to mice (0.2 ml/mouse) every other day for consecutive 4 weeks. Control mice were orally administered with 0.2 ml of distilled water only.

Ehrlich Ascites Tumor (EAT) cells

A line of Ehrlich ascites tumor was supplied through the courtesy of Dr. G. Klien, Amsterdam, Holland. The tumor line was maintained in The Cancer Institute (Cairo, Egypt) in female Swiss albino mice by weekly intraperitoneal transplantation of viable 2x10^6 cells/animal. Tumor cell suspensions were prepared in balanced salt solution at pH 7.4 to a final concentration of 5x10^6 viable cells/ml (Binoletto et al., 2005). All experimental animals were inoculated with EAT cells intraperitoneally (i.p.) in a volume of 0.2 ml (1x10^6 cells) 24 hour after the last injection of honey. One week later, all mice were sacrificed and lymphoid cells, tumor cells as well as sera were obtained for immunological, carcinogenic and biochemical analysis.

Determination of Lymphoid Cell Counts

Normal, EAT-bearing mice, and EAT-bearing mice pretreated with honey were sacrificed, and thymuses (Thy), spleens (Spl), peripheral lymph nodes (PLN) and mesenteric lymph nodes (MLN) were excised, cleaned and weighed. Single cell suspensions were prepared in Hank’s balanced salt solution (HBSS), followed by filtering through a nylon sieve. Bone marrow (BM) was obtained from femurs and tibiae,
suspended in HBSS and filtered. Red blood cells from all these tissues, in addition to peripheral blood (PBl) were lysed by addition of Tris/NaH_2Cl buffer (0.017 M Tris-hydroxymethyl aminomethane and 0.16 M NaHCl, pH 7.2). The respective cell suspensions were washed three times, resuspended in HBSS, and cells were counted with a haemocytometer and calculated per gram of tissue.

Harvesting of Peritoneal Exudates Cells (PEC)

To obtain inflammatory peritoneal phagocytes, normal, EAT-bearing mice and EAT-bearing mice pre-treated with honey were i.p. injected with 2 ml of starch suspension (1% starch in saline). Three days later, mice were sacrificed and the peritoneal exudates cells (PEC) were obtained by peritoneal lavage with 5 ml of HBSS. Cells were washed and resuspended in HBSS. Total and differential counts of PEC were determined using haemocytometer, by the uptake of 1% W/V neutral red in saline (Hudson & Hay, 1989).

Carbon Clearance Assay

The phagocytic activity of PEC was measured by using Pelikan special biological ink (Pelikan-Werke, Hannover, Germany) according to Salem et al. (1996). The original suspension was diluted 1:1 with 0.9% NaCl solution, and 0.2 ml of the diluted ink was i.p. injected into normal, EAT-bearing mice and EAT-bearing mice pre-treated with honey after stimulation with 2 ml of starch suspension, which was i.p. injected three days earlier. Carbon challenged animals were sacrificed 15, 30, 45 and 60 minutes after carbon injection. Five ml of 0.1% EDTA-saline solution was i.p. injected, and the peritoneal lavage was collected and centrifuged at 1200 r.p.m. for 5 min. The resultant supernatant was decanted into another tube, and the precipitated cells were resuspended in 1 ml of equal volumes of gelatin (2% gelatin in saline) and ethanol potassium saline (5% KOH in 70% ethanol), and incubated overnight at 37 °C. Optical densities of both supernatant and digested cells were measured using a spectrophotometer (Spectronic 20, Bausch and Lomb Inc., Rochester, NY, USA).

E-rosette-Forming Cells (RFC) Assay

The procedure was performed as described by Hsu et al. (1975). Seven days before they are sacrificed, the mice received an i.p. injection of 1x10^8 sheep red blood cells (SRBCs) in 0.2 ml saline. Spleens from normal, EAT-bearing mice and EAT-bearing mice pre-treated with honey were excised and cleaned. Single cell suspensions were prepared, washed twice by centrifugation at 1200 r.p.m. for 10 min and resuspended in HBSS to a concentration of 2x10^6. A volume of 0.2 ml of spleen cell suspension was mixed with an equal volume of 0.5% SRBCs in a glass tube and incubated for 2-4 hours at 37 °C. The tubes containing mixture were gently shaken to resuspend the cells in the pellet. The rosettes were counted in haemocytometer and calculated per million mononuclear cells. The cells surrounded by three or more SRBCs were counted as E-rosette-forming T-cells.

Plaque-Forming Cells (PFC) Assay

The procedure was performed as described by Brousseau et al. (1999). Primary humoral immune responses against SRBCs were measured after one i.p. injection of 1x10^5 SRBCs in 0.2 ml saline. Five days later, normal, EAT-bearing mice and EAT-bearing mice pre-treated with honey were sacrificed and spleen were excised and cleaned. Single cell suspensions were prepared, washed twice by centrifugation at 1200 r.p.m. for 10 min and resuspended in HBSS to a concentration of 2x10^6/ml. The liquid assay mixture was prepared by adding 50 µl of 25% SRBCs and 50 µl of guinea pig complement to 100 µl of spleen cell suspension. The assay mixture was plated to a slide chamber and incubated for 30-45 min at 37°C. The plaques were scored microscopically and calculated per million mononuclear cells.

Preparation of Serum

At the end of each experiment, blood was drawn from the abdominal vein in centrifuge tubes and left to clot. Thereafter, blood samples were centrifuged at 3000 r.p.m. for 30 minutes and then serum was separated and kept at -20°C until analysis.

Biochemical Analysis

Total lipids content was determined as described by Zollner et al. (1966) using total lipids kit, LABKIT Co., Spain. Total protein was determined as described by Domas (1975) using protein kit, LABKIT Co., Spain. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were determined as described by Varliy, (1974) using AST and ALT kits, LABKIT Co., Spain. Alkaline phosphatase (ALP) and acid phosphatase (ACP) activities were determined as described by Delfield & Goldberg (1971) using ALP and ACP kits, LABKIT Co., Spain. Urea was determined as described by Weatherbum (1987) using urea kit, LABKIT Co., Spain. Creatinine was determined as described by Kostir & Sonka (1952) using creatinine kit, LABKIT Co., Spain.

Determination of EAT Cell Count

Control mice as well as mice pre-treated with honey were i.p. inoculated with 1x10^6 EAT cells/mouse. One week later, normal, EAT-bearing mice as well as EAT-
bearing mice pre-treated with honey were sacrificed. EAT cells were obtained by peritoneal lavage with 5 ml of HBSS. Cells were washed three times by centrifugation at 1200 r.p.m. for 10 min, resuspended in HBSS, and counted with a haemocytometer.

Viability of EAT Cells

EAT cells (1x10^5, 5x10^5, 1x10^6, 5x10^6 and 1x10^7 cells/well) were seeded into 96-well culture plates (Falcon, Oxnard, CA) in RPMI-1640 medium (100 µl/well). Honey (1, 10 and 100 mg/ml) was added to each EAT cell concentration in a volume of 100 µl/well and incubated overnight at 37 °C. The respective cell suspensions were washed three times, and resuspended in RPMI-1640 medium. The viable cells were counted with a haemocytometer using trypan blue dye. The viability % was calculated according to the following formula:

\[ \text{Viability %} = \frac{\text{No. of viable cells}}{\text{Total N. of cells}} \times 100 \]

Measurement of Solid Tumor

EAT cells were suspended in normal saline and adjusted to a concentration of 20x10^6 cells/ml. 0.2 ml of the cell suspension (4x10^6 cells) was inoculated s.c. in the right thigh of control mice and in mice pre-treated with honey. Palpable tumors were measured after one week using Vernier calipers (Tricle Brand, Shanghai, China). Tumor volume was calculated so as to monitor the response to treatment. According to Papadopoulos et al. (1989), the formula used for this calculation was:

\[ \text{Tumor volume (mm)}^3 = \frac{4 \pi (A/2)^2 (B/2)}{3} \]

Where A is the tumor diameter in the minor axis and B is the tumor diameter in the major axis.

Histological Examination of The Solid Tumor

Tumor-bearing mice as well as tumor-bearing mice pre-treated with honey were sacrificed one week after inoculation of EAT (4x10^6 EAT cells/mouse) in the right thigh of each mouse. The solid tumors were excised and fixed in 10% neutral buffered formalin. The specimens were then dehydrated in ascending grades of ethyl alcohol, cleared in terpinol, washed in benzene, embedded in paraffin wax, sectioned at 5 µ, and stained with haematoxylin and eosin (Delafield, 1984). The stained sections were then examined under light microscopy for assessment of the tumor cell growth using X 160 microscopic magnification.

Statistical Analysis

All in vivo results are expressed as the mean ± SD of groups consisting of 6 mice. The in vitro data are also expressed as the mean ± SD of groups consisting of four wells. Each experiment was performed independently at least three times. All data were analysed for significance using Student’s t-test.

Results

Effect on lymphoid cell count

Table 1 shows that the number of PBl leukocytes from EAT-bearing mice were slightly increased compared to that of normal mice, however, pre-treatment of EAT-bearing mice with honey (10, 100 or 1000 mg/100 g BW, every other day for 4 weeks) elicited a pronounced decrease in the number of PBl leukocytes compared to that of EAT-bearing mice. This decrease was statistically significant with the dose 100 mg (P<0.01). The total number of cells from thymus (P<0.01), PLN and MLN of EAT-bearing mice was decreased, while the total number of cells from Spl was slightly increased compared to those of normal mice. Pre-treatment of EAT-bearing mice with honey caused a significant decrease in the number of splenocytes with doses 10 and 100 mg (P<0.05). BM cells from EAT-bearing mice were significantly decreased when compared with the corresponding value of normal mice. Pre-treatment of EAT-bearing mice with honey caused a statistically significant increase in the total number of BM cells (P<0.01).
### Table 1. Total cell count/gram of tissue in honey treated mice.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PBI x10⁶ (Mean ± SD)</th>
<th>Thy x10⁶</th>
<th>Spl x10⁶</th>
<th>PLN x10⁶</th>
<th>MLN x10⁶</th>
<th>BM x10⁶</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>6.17 ± 1.45</td>
<td>2.05 ± 0.31</td>
<td>2.22 ± 0.29</td>
<td>1.23 ± 0.43</td>
<td>1.04 ± 0.34</td>
<td>2.04 ± 0.34</td>
</tr>
<tr>
<td>Tumor-bearing + Vehicle</td>
<td>7.30 ± 0.99</td>
<td>0.74 ± 0.21**</td>
<td>2.68 ± 0.64</td>
<td>1.19 ± 0.32</td>
<td>0.96 ± 0.25</td>
<td>0.96 ± 0.21##</td>
</tr>
<tr>
<td>Tumor-bearing + Honey (10 mg)</td>
<td>6.00 ± 1.40</td>
<td>0.87 ± 0.28</td>
<td>1.84 ± 0.49*</td>
<td>1.12 ± 0.34</td>
<td>0.98 ± 0.35</td>
<td>2.06 ± 0.46**</td>
</tr>
<tr>
<td>Tumor-bearing + Honey (100 mg)</td>
<td>4.48 ± 1.22**</td>
<td>0.92 ± 0.30</td>
<td>1.78 ± 0.50*</td>
<td>0.98 ± 0.19</td>
<td>0.92 ± 0.29</td>
<td>2.23 ± 0.53**</td>
</tr>
<tr>
<td>Tumor-bearing + Honey (1000 mg)</td>
<td>5.72 ± 1.27</td>
<td>0.89 ± 0.31</td>
<td>1.96 ± 0.52</td>
<td>1.14 ± 0.33</td>
<td>0.99 ± 0.29</td>
<td>1.73 ± 0.37**</td>
</tr>
</tbody>
</table>

Total number of leukocytes from peripheral blood (PBI), as well as cells from thymus (Thy), spleen (Spl), peripheral lymph nodes (PLN), mesenteric lymph nodes (MLN) and bone marrow (BM) were assessed in normal and in tumor-bearing mice pretreated orally with vehicle (0.2 ml distilled water) or honey (10, 100 or 1000 mg/100 g BW) every other day for 4 weeks. (## at P < 0.01 in comparison with the control group; * at P < 0.05 and ** at P < 0.01 in comparison with the tumor-bearing group).

**Effect on Peritoneal Exudate Cell (PEC) Count**

As shown in Table 2, the total number of PEC as well as the absolute number and the relative proportion of lymphocytes of EAT-bearing mice were significantly increased (P<0.01), while the absolute number and the relative proportion of macrophages were significantly decreased (P<0.01) when compared to that of normal mice. Pretreatment of EAT-bearing mice with honey recovered this effect and caused a significant decrease in PEC count as well as the absolute number and the relative proportion of lymphocytes (P<0.01); and a significant increase in the absolute number and the relative proportion of macrophages (P<0.01) with doses 100 and 1000 mg as compared with the corresponding values of the EAT-bearing mice.

**Effect on the Phagocytic Function of Peritoneal Exudate Cells (PECs)**

Table 3 shows that carbon uptake by PECs of EAT-bearing mice was significantly decreased (P<0.01) when compared with that of the normal mice. However, pre-treatment of EAT-bearing mice with honey (10, 100 or 1000 mg/100 g BW, every other day for 4 weeks) caused a progressive increase in the scavenger activity of PECs (P<0.05; P<0.01). On the other hand, the carbon particles remained in the peritoneal fluid of EAT-bearing mice were significantly increased as compared with those of normal mice (P<0.01). Pre-treatment of EAT-bearing mice with honey caused a gradual decrease carbon contents as compared with those of the EAT-bearing control mice (P<0.05; P<0.01).
The anti-tumor effect of bee honey in ehrlich ascite tumor is coincided with stimulation of the immune cells.

Table 2. Total and differential cell count of peritoneal exudates cells in honey treated mice.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total PEC count (Mean ± SD x10^6)</th>
<th>Macrophages</th>
<th>Lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Absolute No. (Mean ± SD x10^6)</td>
<td>%</td>
</tr>
<tr>
<td>Normal</td>
<td>6.41 ± 1.03</td>
<td>4.32 ± 0.49</td>
<td>67.4</td>
</tr>
<tr>
<td>Tumor-bearing + Vehicle</td>
<td>27.89 ± 1.49##</td>
<td>1.58 ± 0.32##</td>
<td>5.7</td>
</tr>
<tr>
<td>Tumor-bearing + Honey (10 mg)</td>
<td>24.22 ± 5.63</td>
<td>2.43 ± 1.09</td>
<td>10.0</td>
</tr>
<tr>
<td>Tumor-bearing + Honey (100 mg)</td>
<td>20.89 ± 3.90**</td>
<td>2.52 ± 0.46**</td>
<td>12.0</td>
</tr>
<tr>
<td>Tumor-bearing + Honey (1000 mg)</td>
<td>12.23 ± 2.77**</td>
<td>3.14 ± 0.59**</td>
<td>25.7</td>
</tr>
</tbody>
</table>

Total peritoneal exudate cells (PEC) count, the absolute number and relative proportion (%) of both macrophages and lymphocytes were assessed in normal and in tumor-bearing mice pre-treated orally with vehicle (0.2 ml distilled water) or honey (10, 100 or 1000 mg/100 g BW) every other day for 4 weeks. (## at P<0.01 in comparison with the control group, and ** at P<0.01 in comparison with the tumor-bearing group).

Table 3. Phagocytic activity of peritoneal exudate cells in honey treated mice.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Carbon uptake by PEC</th>
<th>Carbon particles remained in fluid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>15 min</td>
<td>30 min</td>
</tr>
<tr>
<td>Normal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.85 ± 0.08</td>
<td>1.05 ± 0.07</td>
<td>1.11 ± 0.25</td>
</tr>
<tr>
<td>Tumor-bearing + Vehicle</td>
<td>0.32 ± 0.06##</td>
<td>0.47 ± 0.11##</td>
</tr>
<tr>
<td>0.45 ± 0.13</td>
<td>0.56 ± 0.10</td>
<td>0.78 ± 0.07##</td>
</tr>
<tr>
<td>Tumor-bearing + Honey (10 mg)</td>
<td>0.56 ± 0.10**</td>
<td>0.75 ± 0.08**</td>
</tr>
<tr>
<td>0.61 ± 0.13**</td>
<td>0.68 ± 0.04</td>
<td>0.88 ± 0.06**</td>
</tr>
<tr>
<td>Tumor-bearing + Honey (1000 mg)</td>
<td>0.13 ± 0.11**</td>
<td>0.82 ± 0.04**</td>
</tr>
</tbody>
</table>

The phagocytic activity of peritoneal exudate cells (PEC), as determined by carbon uptake by PEC and carbon particles remained in the peritoneal fluid was assessed in normal and in tumor-bearing mice pre-treated orally with vehicle (0.2 ml distilled water) or honey (10, 100 or 1000 mg/100 g BW) every other day for 4 weeks. (## at P<0.01 in comparison with the control group; * at P<0.05 and ** at P<0.01 in comparison with the tumor-bearing group).

Effect on Rosette-Forming Cells (RFCs) Count

As shown in Table 4, the number of RFCs in EAT-bearing mice was significantly decreased when compared with that of the normal mice (P<0.01). Pre-treatment of EAT-bearing mice with honey (10, 100 or 1000 mg/100 g BW, every other day for 4 weeks) caused a statistically significant increase in the number of RFCs as compared with that of the corresponding EAT-bearing control mice (P<0.01). The increment of this increase reached about 0.56, 1.72 and 1.60 folds respectively.
Effect on Plaque-Forming Cells (PFCs) Count

As shown in Table 4, the number of PFCs in EAT-bearing mice was significantly decreased when compared with that of the normal mice ($P<0.01$). Pre-treatment of EAT-bearing mice with honey (10, 100 or 1000 mg/100 g BW, every other day for 4 weeks) caused a progressive increase in the number of PFCs as compared with that of the corresponding EAT-bearing control mice. However, this increase was statistically significant with doses 100 and 1000 mg ($P<0.05$), and reached about 0.40 and 0.35 folds respectively.

Table 4. T and B lymphocytes in spleen of honey treated mice.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of RFCs/ million nucleated spleen cells (Mean ± SD $\times 10^3$)</th>
<th>No. of PFCs/ million nucleated spleen cells (Mean ± SD $\times 10^3$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>2.45± 0.35</td>
<td>1.42 ± 0.09</td>
</tr>
<tr>
<td>Tumor-bearing + Vehicle</td>
<td>0.87 ± 0.10##</td>
<td>1.10 ± 0.13##</td>
</tr>
<tr>
<td>Tumor-bearing + Honey (10 mg)</td>
<td>1.36 ± 0.09**</td>
<td>1.18 ± 0.17</td>
</tr>
<tr>
<td>Tumor-bearing + Honey (100 mg)</td>
<td>2.37 ± 0.24**</td>
<td>1.54 ± 0.34*</td>
</tr>
<tr>
<td>Tumor-bearing + Honey (1000 mg)</td>
<td>2.26 ± 0.27**</td>
<td>1.48 ± 0.29*</td>
</tr>
</tbody>
</table>

Number of rosette-forming cells (RFCs) and plaque-forming cells (PFCs)/$10^6$ nucleated spleen cells were assessed in normal and in tumor-bearing mice pre-treated orally with vehicle (0.2 ml distilled water) or honey (10, 100 or 1000 mg/100 g BW) every other day for 4 weeks. All mice were immunized i.p. with 0.2 ml of SRBC 4 days before sacrifice. ($##$ at $P<0.01$ in comparison with the control group; * at $P<0.05$ and ** at $P<0.01$ in comparison with the tumor-bearing group).

Effect on Serum Total Lipids and Total Proteins

As shown in Table 5, serum levels of total lipids were decreased, while serum levels of total proteins were increased in EAT-bearing mice as compared with those of normal mice. Pre-treatment of EAT-bearing mice with honey (10, 100 or 1000 mg/100 g BW, every other day for 4 weeks) caused an increase in the serum levels of total lipids and a decrease in the serum levels of total proteins when compared with those of EAT-bearing control mice.

Effect on Serum Enzyme Activities of the Liver

As shown in Table 5, serum enzyme levels of ALT, AST, ALP and ACP of EAT-bearing mice were markedly increased as compared with those of normal mice. Pre-treatment of EAT-bearing mice with honey (10, 100 or 1000 mg/100 g BW, every other day for 4 weeks) ameliorated this effect and caused a progressive decrease in serum enzyme levels of the liver as compared with those of the EAT-bearing control mice.
The anti-tumor effect of bee honey in ehrlich ascite tumor is coincided with stimulation of the immune cells.

Table 5. Serum chemistry profile in honey treated mice.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total lipids</th>
<th>Total protein</th>
<th>ALT</th>
<th>AST</th>
<th>ALP</th>
<th>ACP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ref. Range: 0.4-1.0 g/dl</td>
<td>Ref. Range: 6.5-8.3 g/dl</td>
<td>Ref Range: up to 32 U/L</td>
<td>Ref. Range: up to 31 U/L</td>
<td>Ref. Range: 98-279 U/L</td>
<td>Ref. Range: up to 11 U/L</td>
</tr>
<tr>
<td>Normal</td>
<td>0.8</td>
<td>6.2</td>
<td>10</td>
<td>63</td>
<td>237</td>
<td>3.7</td>
</tr>
<tr>
<td>Tumor-bearing + Vehicle</td>
<td>0.38</td>
<td>8.5</td>
<td>52</td>
<td>230</td>
<td>292</td>
<td>23</td>
</tr>
<tr>
<td>Tumor-bearing + Honey (10 mg)</td>
<td>0.42</td>
<td>6.5</td>
<td>15</td>
<td>220</td>
<td>134</td>
<td>18</td>
</tr>
<tr>
<td>Tumor-bearing + Honey (100 mg)</td>
<td>0.56</td>
<td>7.5</td>
<td>10</td>
<td>115</td>
<td>192</td>
<td>11</td>
</tr>
<tr>
<td>Tumor-bearing + Honey (1000 mg)</td>
<td>0.40</td>
<td>7.6</td>
<td>10</td>
<td>134</td>
<td>296</td>
<td>21</td>
</tr>
</tbody>
</table>

Serum levels of total lipids, total proteins, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and acid phosphatase (ACP) were assessed in normal and in tumor-bearing mice pre-treated orally with vehicle (0.2 ml distilled water) or honey (10, 100 or 1000 mg/100 g BW) every other day for 4 weeks.

Effect on Serum Enzyme Activities of the Kidney

As shown in Table 6, serum urea and creatinine levels of EAT-bearing mice were increased as compared with those of normal mice. However, pre-treatment of EAT-bearing mice with honey (10, 100 or 1000 mg/100 g BW, every other day for 4 weeks) ameliorated this effect and caused a progressive decrease in serum urea and creatinine levels as compared with those of the EAT-bearing control mice.

Table 6. Serum urea and creatinine in honey treated mice.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Urea (Ref. range: 15-45 mg/dl)</th>
<th>Creatinine (Ref. range: 0.5-1.4 mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>33</td>
<td>0.9</td>
</tr>
<tr>
<td>Tumor-bearing + Vehicle</td>
<td>55</td>
<td>1.1</td>
</tr>
<tr>
<td>Tumor-bearing + Honey (10 mg)</td>
<td>48</td>
<td>1.2</td>
</tr>
<tr>
<td>Tumor-bearing + Honey (100 mg)</td>
<td>40</td>
<td>0.8</td>
</tr>
<tr>
<td>Tumor-bearing + Honey (1000 mg)</td>
<td>18</td>
<td>0.6</td>
</tr>
</tbody>
</table>

Normal and tumor-bearing mice were pre-treated orally with vehicle (0.2 ml distilled water) or honey (10, 100 or 1000 mg/100 g BW) every other day for 4 weeks.
Effect on EAT Cell Count
As shown in Table 7, the number of EAT cells one week after incubation in the abdominal cavity of mice pre-treated with honey (10, 100 or 1000 mg/100 g BW, every other day for 4 weeks) was progressively decreased as compared with that of the corresponding vehicle-treated control mice. However, this decrease was statistically significant with doses 100 and 1000 mg ($P<0.01$).

Table 7. Number of tumor cells in honey treated mice.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of tumor cells (Mean ± SD x10$^6$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>24.12 ± 2.92</td>
</tr>
<tr>
<td>Honey (10 mg)</td>
<td>19.84 ± 3.99</td>
</tr>
<tr>
<td>Honey (100 mg)</td>
<td>16.22 ± 3.93##</td>
</tr>
<tr>
<td>Honey (1000 mg)</td>
<td>9.77 ± 2.98##</td>
</tr>
</tbody>
</table>

Number of tumor cells was assessed one week after incubation of 1x10$^6$ tumor cells in the abdominal cavity of mice pre-treated orally with vehicle (0.2 ml distilled water) or honey (10, 100 or 1000 mg/100 g BW) every other day for 4 weeks. (## at $P < 0.01$ in comparison with the control group).

Effect on the Viability of EAT Cells *in vitro*
As shown in Table 8, *in vitro* incubation of honey (1, 10 or 100 mg/ml) with serial concentrations of EAT cells (1x10$^5$, 5x10$^5$, 1x10$^6$, 5x10$^6$ and 1x10$^7$) for 24 hours elicited a progressive decrease in the % of viable tumor cells as compared with those of vehicle control.

Table 8. Tumor cell viability in honey treated mice.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% of viable tumor cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1x10$^5$</td>
</tr>
<tr>
<td>Vehicle</td>
<td>92</td>
</tr>
<tr>
<td>Honey (1 mg/ml)</td>
<td>78</td>
</tr>
<tr>
<td>Honey (10 mg/ml)</td>
<td>67</td>
</tr>
<tr>
<td>Honey (100 mg/ml)</td>
<td>72</td>
</tr>
</tbody>
</table>

Viability % of tumor cells was assessed *in vitro*. Serial concentrations of tumor cells (1x10$^5$-1x10$^7$ cells) were exposed to vehicle (100 µl RPMI medium) or honey (1, 10 or 100 mg/ml) and incubated for 24 hours.

Effect on the Volume of Solid Tumor
As shown in Table 9, the volume of solid Ehrlich tumor of mice pre-treated with honey (10, 100 or 1000 mg/100 g BW, every other day for 4 weeks) was markedly decreased when compared with that of vehicle-treated control group. The percentage of decrease reached about 19.5, 66.2 and 84.9 % respectively.

Histological Architecture of the Solid Tumor Mass
Administration of EAT cells within the thigh muscles of mice resulted in proliferation and growth of the tumor cells that form tumor masses infiltrating the muscle fibers (Fig. 1 a). A gradual reduction in the tumor cell masses was noticed when mice were pre-treated with honey with doses 10, 100 and 1000 mg/100g BW (Fig. 1 b-d) respectively.
The anti-tumor effect of bee honey in ehrlich ascite tumor is coincided with stimulation of the immune cells.

Table 9. Size of tumor in honey treated mice.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Measurement of tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(A) Minor axis (mm)</td>
</tr>
<tr>
<td>Tumor-bearing + Vehicle</td>
<td>2.0</td>
</tr>
<tr>
<td>Tumor-bearing + Honey (10 mg)</td>
<td>2.0</td>
</tr>
<tr>
<td>Tumor-bearing + Honey (100 mg)</td>
<td>1.5</td>
</tr>
<tr>
<td>Tumor-bearing + Honey (1000 mg)</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Percentage of solid Ehrlich carcinoma growth was assessed in mice pre-treated orally with honey (10, 100 or 1000 mg/100 g BW) every other day for 4 weeks, compared to the tumor-bearing control group. Tumor volume (mm$^3$) = $\frac{4 \pi (A/2)^2 \times (B/2)}{3}$

Figure 1. (a) Longitudinal section of the thigh muscles of EAT-bearing mice showing the proliferation and growth of the tumor cells. (b-d) Longitudinal sections of the thigh muscles of tumor-bearing mice pre-treated with honey (10, 100 and 1000 mg/100g BW) showing the marked reduction of the tumor cell masses. (H&E, X 160).
Discussion
The present study showed that honey pretreatment (10, 100 or 1000 mg/100 g BW) every other day for 4 weeks to EAT-bearing mice significantly increased the number of BM lymphocytes as well as peritoneal macrophages, but not peripheral blood lymphocytes nor splenocytes. The phagocytic function of macrophages as well as T cell and B cell activities were significantly increased, indicating that the preventive treatment with honey could activate the immune system. These results are consistent with the results by Karmakar et al. (2004) who found that honey significantly increased peritoneal macrophages but significantly decreased splenic lymphocyte count. However, Orsolic & Basic (2004) have observed an increase in the number of peripheral blood leukocytes and increased activity of peritoneal macrophages.

It is well known that macrophages are the major factor of host defense. Macrophages from mammary tumor-bearing mice have impaired cytotoxic activity against syngeneic tumor target (Sotomayor et al., 1995). Tumor-associated macrophages were shown to possess tumor growth-promoting abilities that stimulate tumor growth and, in addition, these macrophages suppress many T cell and NK cell antitumor responses (Kono et al., 1996). The present results are in relatively good agreement with the findings described above and suggest that the preventive treatment with honey could reduce tumor growth by activating macrophage function.

The present study showed a gradual increase in PFC response in honey pre-treated EAT-bearing mice, immunized with 1x10^8 SRBCs 7 days before sacrifice, as compared with that of the EAT-bearing control mice. These results agree with the results by Al-Waili & Haq (2004) who first reported that honey increased antibody production during primary and secondary immune responses against thymus-dependent antigen (SRBCs). The present results are also consistent with the results by Karmakar et al. (2004) who reported that honey significantly increased anti-SRBC antibody titers in immunocompetent mice.

The actual mechanism to stimulate antibody production was not identified. However, Al-Waili & Boni (2003) have found that honey increased salivary nitric oxide in humans. Also, honey given to sheep, increased plasma and urinary nitric oxide levels (Al-Waili, 2003a). Nitric oxide is a very important mediator of immune responses (Zeidek & Masek, 1998). It inhibits tumor growth and metastasis (Zeidek & Masek, 1998). A single dose of L-arginine, a known precursor of nitric oxide, caused a significant increase in humoral immune response (Sunita et al., 2000). Therefore, honey might increase humoral immunity by means of enhancing nitric oxide production.

The present data showed that honey pretreatment to EAT-bearing mice recovered the total lipids, total protein, ALT, AST, ALP, ACP and urea and creatinine levels as compared to those of tumor-bearing control mice. These results confirm the results of Al-Waili et al (2003b, c) who reported that slow i.v. infusion or rapid i.v. injection of honey in different concentrations was safe and could improve renal and hepatic functions as well as lipid profile. Honey decreased AST by 22 %, ALT by 18 %, and creatinine kinase by 33 %. The present results also confirm the results of Al-Waili et al. (2006) who reported a significant reduction in AST, ALT and alkaline phosphatase and, and a significant elevation of serum albumin with honey feeding.

Several cellular components such as polyamines and polyamine synthetic enzyme activities including ornithine decarboxylase are present at high levels in proliferating
neoplastic cells (Heby, 1981; Spector & Moore, 1988). In addition, many kinases, such as tyrosine protein kinase, mediate proliferative as well as metabolic signals in the cells. Eicosanoids, the metabolites of arachidonic acid through the lipo-oxygenase and cyclo-oxygenase pathways, exerts a variety of biological activities (Wattenberg et al., 1980; Honn et al., 1989). The mechanism of antitumor effect shown in the present study may be related to the inhibitory effect of honey on tyrosine protein kinase, lipo-oxygenase and cyclo-oxygenase pathways metabolites.

The present results clearly demonstrated the inhibitory effect of honey on EAT cell proliferation in the abdominal cavity of mice, on the viability % of tumor cells as well as on the size of solid tumor. These results are consistent with the results by Swellam et al. (2003) who found that is an effective agent for inhibiting the growth of T24, RT4, 253J and MBT-2 bladder cancer cell lines in vitro. It is also effective in the MBT-2 bladder cancer implantation model. The present results also agree with the results by Orsolic & Basic (2004) who demonstrated the inhibitory effect of honey on metastasis formation of a mammary carcinoma and fibrosarcoma in CBA mice as well as on adenocarcinoma of Y59 rats.

Honey contains a variety of compounds including caffeic acid, benzoic acid and esters, substituted phenolic acid and esters, flavonoids glycones, and bee wax (Heby, 1981; Greenaway et al., 1987). Some of the observed biological activities of honey may be traced to its chemical constituents (Spector & Moore, 1981; Honn et al., 1989). It is likely that, polyphenolic components present in honey stimulate host antitumor defense (Orsolic & Basic, 2003). Wattenberg et al. (1980) demonstrated that dietary administration of hydroxycinnamates (constituents of honey) significantly inhibited benzopyrene-induced neoplasia of the forestomach in mice. Caffeic acid ester derivatives are thought to exhibit a broad spectrum of activities that possibly include tumor inhibition (Chinthalapally et al., 1993).

Apoptosis of tumor cells suggests one of the ways by which honey induces cellular death. Marked cellular and nuclear changes suggestive of apoptosis were observed in honey-treated cancer cell lines (Swellam et al., 2003). By measuring the DNA content of cancer cell lines, both ploidy and S-phase fractions have a prognostic significance in bladder cancer. Treatment of bladder cancer cell lines with honey resulted in a significant reduction in the S-phase fraction, and the accumulation of large cell population behind the G1 peak might be an indicator for apoptosis (Swellam et al., 2003).

In conclusion, the present results indicate that honey treatment to mice is considerably effective against EAT model in vivo and in vitro. Its antitumor activity may occur through the activation of macrophages, T cells and B-cells. The property of honey to stimulate both humeral and cell-mediated immunity will expand its clinical application and explain some of its biological activities, particularly in host defense against tumors.

References


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